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| AD840536   |
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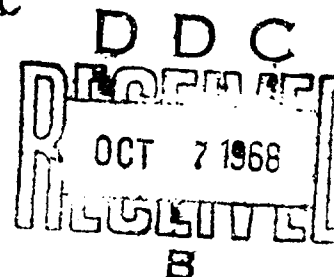
TRANSLATION NO. 2196

DATE: 9 May 1966

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DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

LABORATORY DIAGNOSIS OF SMALLPOX AT THE  
INSTITUTE OF MARINE MEDICINE IN GDANSK

Bul Inst Mar Med Gdansk  
(Bulletin of the Institute of Marine Medicine in Gdansk)  
15, 1964, pages 23-28

Blawat F.

The Virology Laboratory at the Institute participated in a laboratory diagnosis of smallpox during the epidemic which broke out in Poland during the third quarter of 1963. Specimens were sent to this laboratory from 17 July through 16 September, initially from suspected cases throughout the country, but on 6 September the authorities organized operations on a regional basis and instructed that the Institute be sent specimens taken from confirmed and suspect smallpox patients from the six closest wojewodztwos.

The materials were received in 32 shipments containing 74 specimens. They were brought by special couriers by rail, and in some cases by health service airplane. They were sent by wojewodztwo or municipal health-epidemiologic stations or hospitals, from the following localities: Wroclaw, Lodz (Wieruszow), Warsaw, Rzeszow, Szczecin, Zlotow, Gdansk, Bydgoszcz.

A lack of experience was surely the reason for the fact that the specimens were sometimes taken improperly or in insufficient quantity, or poorly packaged. After the Health-Epidemiologic Department of the Ministry of Health and Social Security issued appropriate instructions on 8 September the situation improved considerably. We see as a major defect the absence in the overwhelming majority of specimen shipments of brief information on the patients from whom the specimens had been taken. We were also very seldom informed of the stage of the disease at which the specimens were taken.

DIAGNOSTIC METHODS EMPLOYED

General Remarks

We employed the majority or only a few of the following methods, depending on the quantity and quality of specimen: (1) direct microscope examination of the specimen, stained according to Gutstein's method; (2) direct

examination of the specimen in the electron microscope; (3) examination on a developing chicken embryo by chorio-allantois seeding (CAM); (4) check on CAM results; (a) in the electron microscope; (b) in gel precipitation reaction (Dumbell-Nizamuddin, 1959); (c) in replenisher binding reaction; (d) in hemagglutination reaction; (e) in hemagglutination inhibition reaction; (5) examination in tissue culture through seeding on FL cell cultures.

We did not subject the serologic material to direct examination for the presence of specific antigen, since for the most part the specimens were too meager for us to expect favorable results. We did not receive blood samples to test for the presence of antibodies. The four blood specimens we did receive had been hemolyzed (as a result of being sent together with other specimens in an ice-filled thermos).

In principle all specimens were seeded on the CAM of 11-13 day old chicken embryos, on a culture of FL cells, and the CAM results were verified with the electron microscope. Skin eruption material, when the specimen was sufficient, was examined directly in the electron microscope. Positive and doubtful results from CAM or tissue culture isolation were given supplementary serologic examination.

#### Detailed Description of Procedures

1. Direct preparation of materials for photography in electron microscope. Zeiss Jena D2 microscope, 1958.

Preparations were made from the materials received, based on the methods described by Peters and Neseman [4] as well as Kunert [3], in a portable box in the presence of a burning quartz lamp. We employed here skin change specimens sent in a capillary tube, in the form of slide or swab smears. The dried specimens were suspended on a glass slide in a small quantity of double distilled water or 0.85% NaCl. The moistened material was applied a collodion film and placed on a microscope reticle. The specimen slides were then allowed to dry (about five minutes). We then fixed the preparation, placing the slides into a Petri dish and applying several drops of 2% osmic acid on the upper inside part of the dish. After five minutes the fixed preparations were washed twice with double-distilled water in such a manner that a drop of water would be carefully applied to the film with a Pasteur pipet and then removed with blotting paper. The finished preparations were chromium dusted.

2. Preparation of CAM material for electron microscope photography.

Suspected spots (foci) were pricked with a dull-pointed preparatory needle until completely "melting away". These sites were contacted by collodion film on a specimen slide; excess material was removed with blotting paper. After the added material dried the preparation was twice washed with double-distilled water. A drop of 0.15% pancreatin solution in PBS was then added to the preparation. After five minutes the pancreatin

solution was removed from the edge of the reticle with blotting paper, the preparation was allowed to dry once more, and again washed with double-distilled water and fixed with osmic acid as described above. After fixing we washed it twice in water and dusted with chromium. We should like to emphasize that the introduction of an innovation, based on brief pancreatin etching of the preparation placed on the preparation grid produces images which are more legible and easier to interpret. If this step is bypassed the CAM preparations are overcharged with tissue material and the typical brick-like shape of the smallpox virus is less well-defined.

3. Preparation of the specimens and seeding on the chorioallantois of a developing chicken embryo were handled in a normal way. Crusts were ground in a Weigel mortar. A specimen of the material to be examined was suspended in one ml PBS with the addition of 5,000 units of penicillin and 1,000 mcg of streptomycin [1], refrigerated (+4°C) or left at room temperature for 30 minutes. Three additional dilutions were prepared in a PBS solution without antibiotics, transferring one or two drops of suspension from the first test tube to the second, etc., obtaining dilutions approximating  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ . 0.1 ml of each dilution was introduced to the CAM of 3-4 chicken embryos. On the third day we verified the results of CAM infection macroscopically and by investigating the membrane material in the electron microscope. When necessary we ran it through a second and third time on the CAM. In positive and doubtful cases the CAM results were verified by serologic examination.

The gel precipitation reaction was obtained by the Ouchterlona method after K. R. Dumbell and M. D. Nizamuddin [2]. We employed human test serum obtained from the Statens Serum Institute in Copenhagen and rabbit immune serum prepared in the laboratory.

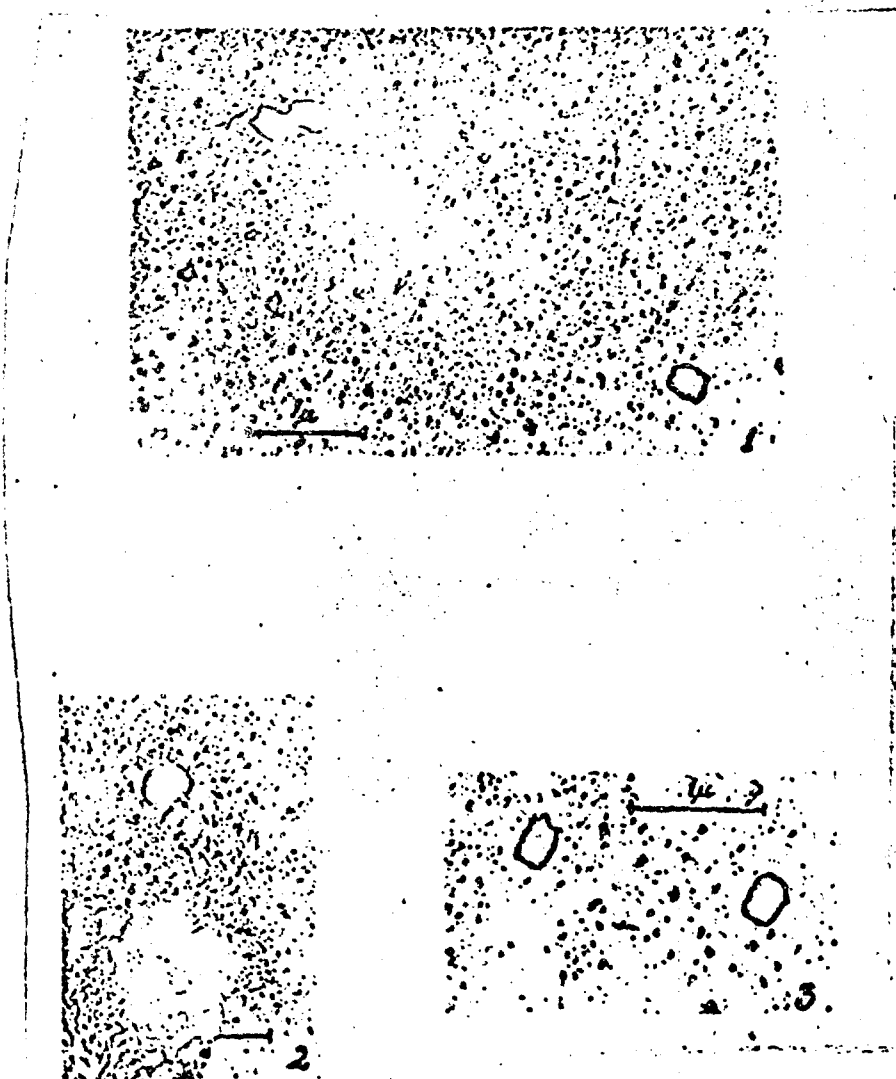
The replenisher binding reaction was effected on plexiglass plates according to the instructions of the Central Public Health Laboratory, London-Colindale.

The hemagglutination and hemagglutination inhibition reaction were obtained according to accepted methods.

Contemporaneously with the embryo tests we seeded on a 2-3 day cell culture of human amnion FL in Leighton test tubes, employing as supporting fluid Eagle's fluid with an addition of 2% calf serum. The culture was observed for 12 days. After the appearance of plaques we removed the cell culture cover glass and stained with hematoxylin and eosin. Positive cultures were verified with CAM and gel precipitation reaction.

## RESULTS AND DISCUSSION

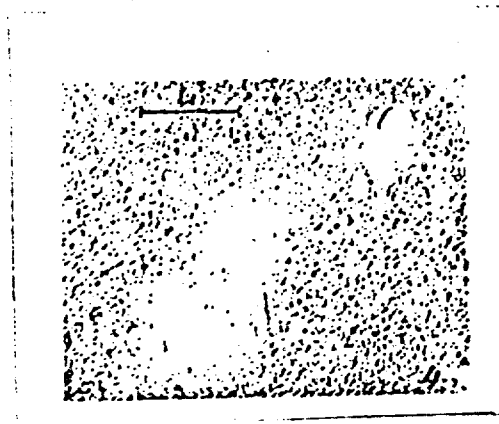
Table 1 presents a compilation of test specimens received together with examination results.



Photographs 1 and 3. Smallpox Virus. Photograph of Direct Specimen (Case LW), Taken Through Electron Microscope.  
Photograph 2. Smallpox Virus. Photograph of Direct Specimen (Case W 2), Taken Through Electron Microscope

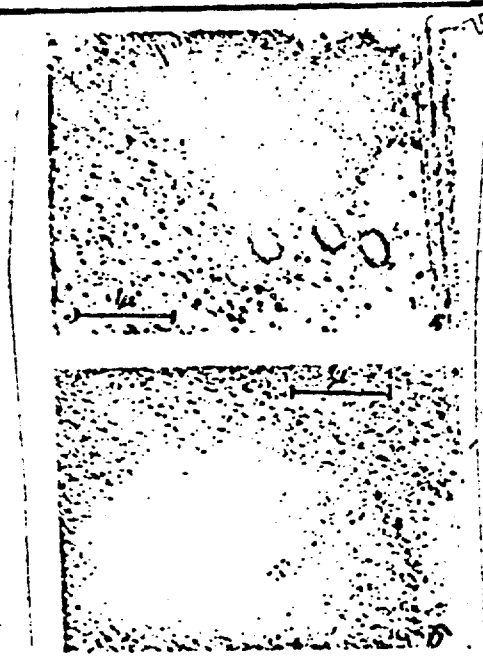
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Photograph 4. Smallpox Virus. Photograph of Direct Specimen (Case W 2), Taken through Electron Microscope.

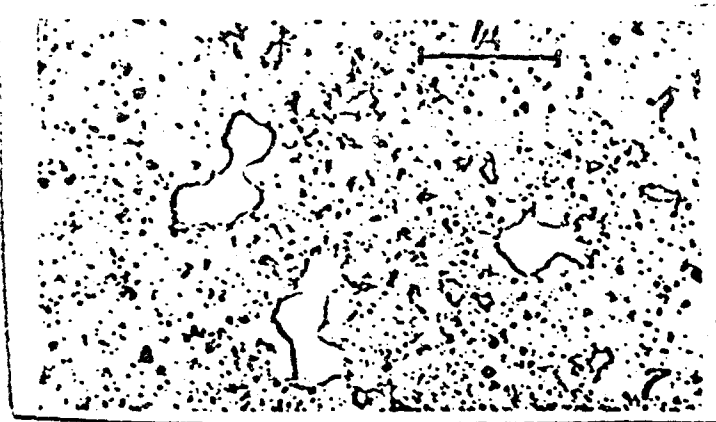
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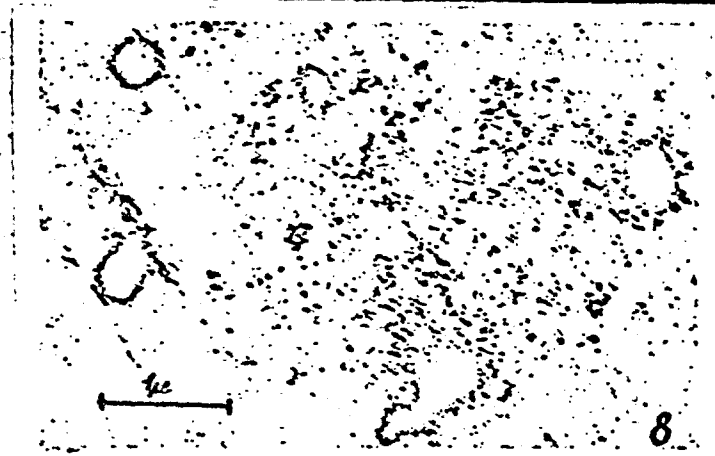
Photographs 5 and 6. Smallpox Virus. Photographs of direct specimen (Case W 2) taken through electron microscope.

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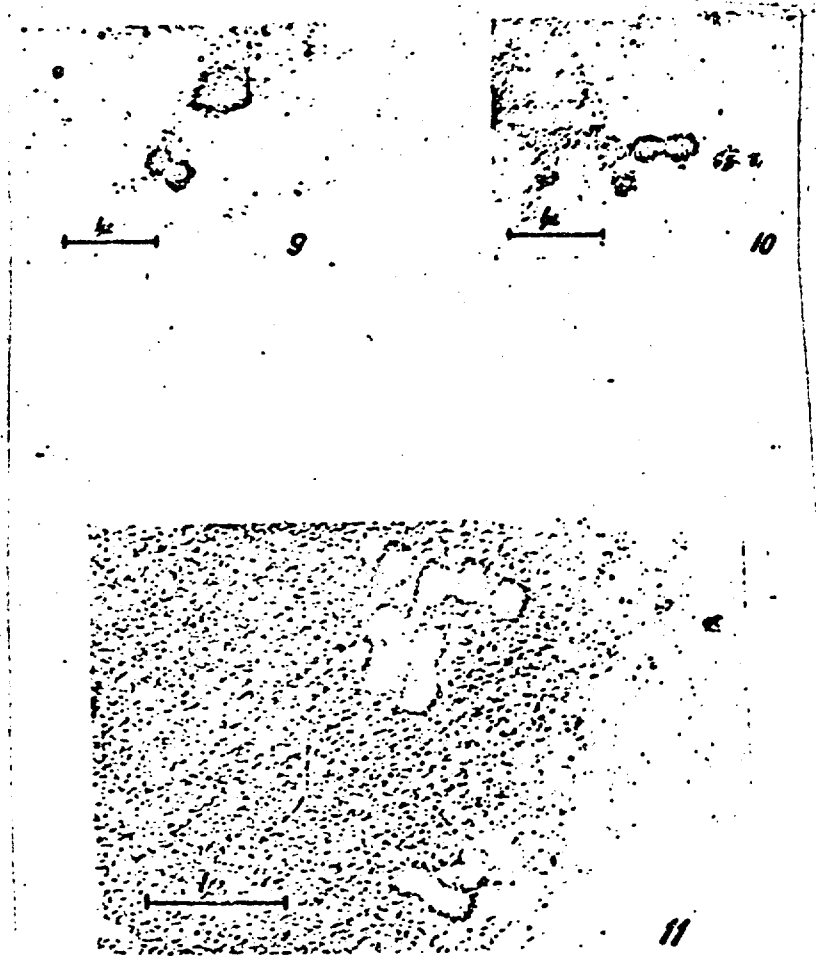
Photograph 7. Smallpox Virus. Photograph of Direct Specimen (Case LW), Taken Through Electron Microscope.



Photograph 8. Smallpox Virus. Photograph from Infected CAM (Case LW), Taken through Electron Microscope

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Photographs 9, 10 and 11. Smallpox Virus. Photographs of Infected CAM  
(Case W 1), Taken through Electron Microscope

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Table 1

Results of Virologic examination of received test specimens for determining smallpox.

| Rodzaj prób 10  | Liczba prób  |           | Wynik badania |         | 13 Wirus |            |
|---|--------------|-----------|---------------|---------|----------|------------|
|   | nadesła-nych | bada-nych | dodatni       | ujem-ny | ospy     | krowian-ki |
|   | 14           | 15        | 16            | 17      | 18       | 19         |
| 1. Rozmaz treści pęcherzyków lub krost na szkiełku                    | 14           | 14        | 7             | 7       | 5        | 2          |
| 2. Treść pęcherzyków lub krost w probówkach wzgl. rurkach włosowatych | 14           | 14        | 4             | 10      | 2        | 2          |
| 3. Strupki  | 10           | 10        | 2             | 8       | 2        | 0          |
| 4. Zeskrobiny   | 2            | 2         | 1             | 2       | 1        | 0          |
| 5. Wymaz treści pęcherzyków lub krost na waciku                       | 2            | 2         | 2             | 0       | 0        | 2          |
| 6. Wymaz z gardła na waciku   | 22           | 20        | 0             | 20      | 0        | 0          |
| 7. Popłuczyny z gardła  | 5            | 2         | 0             | 2       | 0        | 0          |
| 8. Krew skrzepnięta zhemolizowana (w lodzie)                          | 4            | 2         | 0             | 2       | 0        | 0          |
| 9. Razem  | 74           | 68        | 10            | 52      | 10       | 0          |

Key: 1 -- smear of vesicle or pustule contents on glass; 2 -- contents of vesicles or pustules in test tubes or capillary tubes; 3 -- crusts; 4 -- scrapings; 5 -- swab of vesicle or pustule contents; 6 -- pharyngeal swab; 7 -- pharyngeal washings; 8 -- hemolyzed in ice (coagulated blood; 9 -- total; 10 -- type of specimen; 11 -- number of specimens; 12 -- result of examination; 13 -- virus; 14 -- received; 15 -- examined; 16 -- positive; 17 -- negative; 18 -- smallpox; 19 -- vaccinia

Sixty-eight of 74 specimens were examined; the remaining six were either not suited for examination or examination would have been inappropriate. Sixteen of 43 skin eruption specimens examined showed the presence of viruses of the smallpox group: smallpox virus in 10 specimens and vaccinia virus in six. The remaining specimens tested negative. The above results of positive-testing specimens enabled us to confirm infection by smallpox virus in six individuals and vaccinia virus in three.

The electron microscope was a valuable aid in our laboratory diagnosis. We used it to examine direct specimens from skin eruption and obtained rough results within two to four hours. As mentioned above, in some cases there was only one specimen and it was used up entirely for the primary examinations, that is for virus propagation in chicken embryo and tissue culture. It is therefore impossible to draw comparative conclusions from direct specimen examination in the electron microscope. We used the electron microscope to examine 26 direct samples, seven of which were morphologically positive, that is indicated characteristic elementary bodies, with 11 samples virologically positive. The electron microscope was quite useful in examining CAM with non-typical eruptions. The attached plates present some pictures of smallpox viruses obtained in examining direct specimens as well as infected CAM.

Examination of eruptions specimens from stricken individuals from the smallpox epidemic area enabled us to establish laboratory identification relatively rapidly, employing the above methods. But discovery of the virus in subsequent waves of infection in an area of partial immunity, particularly in abortive cases, presents a more difficult problem, as has been demonstrated by the most recent European epidemics.

#### Bibliography

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